The role of abca1 in atherosclerosis: lessons from in vitro and in vivo models
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Chapter 4

Increased ABCA1 activity protects against atherosclerosis

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Abstract
The ABC transporter ABCA1 plays a key role in the first steps of the reverse cholesterol transport pathway by mediating lipid efflux from macrophages. Previously, it was demonstrated that human ABCA1 overexpression in vivo in transgenic mice results in a mild elevation of plasma HDL levels and increased efflux of cholesterol from macrophages. In this study we determined the effect of overexpression of ABCA1 on atherosclerosis development. Human ABCA1 transgenic mice (BAC+) were crossed with ApoE-/− mice, a strain which spontaneously develop atherosclerotic lesions. BAC+ApoE-/− mice exhibited a dramatic reduction in lesion size, containing early, less complex lesions as compared with their ApoE-/− counterparts. In addition, there was increased efflux of cholesterol from macrophages isolated from the BAC+ApoE-/− mice. Although the increase in plasma HDL cholesterol levels was small, HDL particles from BAC+ApoE-/− mice were significantly better acceptors of cholesterol. Lipid analysis of HDL particles from BAC+ApoE-/− mice revealed an increase in phospholipid levels, which was correlated significantly with their ability to enhance cholesterol efflux. We conclude that raising ABCA1 activity in vivo results in significant protection against atherosclerosis.
**Introduction**

The inverse relationship between serum concentration of high density lipoprotein cholesterol (HDL-C) and apolipoprotein Al (ApoA-I), with coronary heart disease is well established (1). The ability to promote reverse cholesterol transport is accepted as one important mechanism by which HDL can protect against atherosclerosis(2). Reverse cholesterol transport is the process by which cholesterol is removed from extrahepatic tissues and returned to the liver for conversion into bile acids and excretion into bile. Inhibition of the oxidative modification of low density lipoprotein (LDL) (3), and down-regulation of the cytokine-induced expression of adhesion molecules in endothelial cells (4) are other mechanisms proposed to explain the atheroprotective actions of HDL.

The presence of macrophage derived foam cells is one of the characteristic features of the early stages of atherosclerotic lesion formation (5,6). Efficient cholesterol efflux from macrophages is critical for the prevention of foam cell formation and subsequent protection against atherosclerosis. The first crucial step in the reverse cholesterol transport pathway is the movement of excess cellular cholesterol and phospholipid from cell membranes to nascent HDL particles (7;8). The adenosine triphosphate-binding cassette transporter A1 (ABCA1) plays a key role in this process, although the biochemical mechanisms involved remain to be elucidated. One model proposes that rather than interacting directly with cholesterol, ABCA1 facilitates the translocation of phospholipids to free ApoA-I at the plasma membrane. The phospholipid-ApoA-I complexes thus formed may subsequently stimulate the efflux of cholesterol in a secondary fashion (9;10). Alternatively, ABCA1 may act as a cholesterol/phospholipid flippase at the plasma membrane, since it stimulates phospholipid and cholesterol efflux to ApoA-I (11,12). Disruption in ABCA1 function due to mutations in the *ABCA1* gene cause Tangier disease and familial hypoalphalipoproteinemia (FHA) which are characterized by low to absent HDL levels, and an increased deposition of cholesteryl esters in several tissues and cells, most notably in macrophages (13). A 50% reduction in ABCA1 activity is associated with a significant decrease in plasma HDL cholesterol levels (14,15,16).

To study the role of ABCA1 in HDL metabolism and atherosclerosis, we have developed human ABCA1 overexpressing BAC transgenic mice that show increased plasma total cholesterol and HDL-C levels, and increased ApoA-I and ApoA II levels (17) associated with increased cholesterol efflux from macrophages. We hypothesized that increasing ABCA1 activity would be associated with reduced atherosclerotic lesion formation.

ApoE-/- mice have been used extensively to study atherogenesis. These mice spontaneously develop severe atherosclerosis, with complex lesions that include a fibrous cap in mice as young as 15 weeks of age. Because of its validation and acceptance as a model to study factors influencing atherogenesis, we chose to test the effect of ABCA1 overexpression on atherosclerosis development, macrophage cholesterol efflux and HDL composition in this model.
Materials and methods

Generation of ABCA1 BAC+ApoE/- mice

The generation of the ABCA1 BAC transgenic mice has been described previously (17). ABCA1 BAC transgenic mice hemizygous for the human ABCA1 gene and backcrossed to C57BL/6J (N3) were crossed to ApoE/- mice on a pure C57BL/6 background (C57BL/6J Apoe-/-). The resulting BAC+ ApoE+/+ mice were backcrossed once more to the ApoE/- mice, and the BAC+ ApoE/- offspring were then backcrossed again to ApoE/- mice, generating BAC+ ApoE/- and BAC- ApoE/- (henceforth called ApoE/-) control littermates that were backcrossed to the N6 generation (98.4%) on the C57BL/6J background. All experiments were performed with the approval of the Animal Care Committee of UBC.

Histological manipulations of aortas

For the analysis of aortic lesions, 12 week old BAC+ApoE/- and ApoE/- littermate controls kept on a chow diet were fasted for 4 hours, followed by injection of 400μl of 0.02g/ml Avertin (Aldrich). The chow diet was a 1:1 mix of Picolab Mouse Diet 20 and Picolab Rodent Diet 20, containing 9% and 4.5% fat respectively. Tissues were harvested for RNA and protein analysis. Mice were perfused transcardially with 4% paraformaldehyde (PFA)(Fisher) and the hearts with attached aortas were isolated. Tissues were embedded in Optimal Cutting Temperature (OCT) (TissueTek) media in a plastic mold, frozen and sixteen 10μm sections were cut. For Oil Red O (ORO) staining, the sections were rinsed in water and isopropanol, and stained in 0.25% ORO for 20 minutes, followed by an isopropanol rinse. Sections were counterstained in Gill’s hematoxylin for 1 minute, and mounted. For Movat’s pentachrome staining, sections were hydrated, stained in 1% Alcian blue for 30 minutes, and rinsed in 1% acetic acid. Sections were then post-fixed in formol-sublimate fixative overnight, rinsed, and treated with Lugol’s iodine for 4 minutes. Sections were treated with 5% sodium thiosulfate, stained in Verhoeff’s hematoxylin, and differentiated in 2% ferric chloride. Sections were air dried, stained in 1.5% saffron for 1 minute, dehydrated in 100% ethanol and mounted. Transverse sections were obtained from the apex of the heart moving toward the aortic region, with sections beginning at the point where all three aortic valve cusps became clearly visible. Every fourth section was placed on a slide for ORO staining of neutral lipid, and counter stained with hematoxylin, such that each slide had four sections 40μm apart, as previously described (18). Sections adjacent to those stained with ORO were stained with Movat’s pentachrome for the identification of elastin, collagen, glycosaminoglycans, smooth muscle cells and foam cells.

Lesion assessment

Assessment of lesions was performed as described previously (18). Lesion areas were photographed using an Axioskop2 (Zeiss) microscope, with a SPOT (Diagnostic Instruments, Inc.) camera, and Northern Eclipse software (Empix Imaging) was used for quantitation of ORO
positive areas within the lesion sites. The total ORO staining area within the sinuses of valsalva of four sections were averaged to provide the lesion area for each mouse.

Protein analysis
To ensure that the tissue distribution of ABCA1 protein had not been altered in the absence of the ApoE gene, we isolated proteins from various tissues and subjected them to Western blot analysis. Tissue extracts were prepared as previously described (17). Total protein was transferred to a polyvinylidene fluoride (PVDF) (Millipore) membrane and Western immunoblotting was performed with a primary monoclonal anti-ABCA1 antibody raised against the second nucleotide binding domain (NBD2) of human ABCA1 (19). Blots were probed with anti-GAPDH (Sigma-Aldrich) to ensure equal protein loading.

Expression of human specific ABCA1 transcript
To definitively determine if the human BAC is expressed in the mice, we performed human ABCA1 specific RT-PCR analysis. PCR primers and conditions were as described previously (17).

Efflux of ³H-cholesterol from primary macrophages
Mice were injected with 2ml of 3% thioglycollate broth. Three days later, peritoneal macrophages were isolated and plated at a density of 5x10⁵ cells/mL into each of 6 wells of a 24 well plate. [³H]-cholesterol (2µCi/ml) (Perkin Elmer Life Sciences) and 50µg/ml acylated low density lipoprotein (Intracel) were preincubated at 37°C for 30 minutes, and subsequently added to DMEM containing 1% FBS, penicillin/streptomycin, l-glutamine, and 1µM acyl-CoA:cholesterol O-acyltransferase (ACAT) inhibitor (CI 976, a kind gift from Dr. Minghan Wang, Pfizer Global Research). The addition of the ACAT inhibitor decreases the levels of cholesterol esterification in macrophages, and therefore facilitates free cholesterol efflux out of the cells (20). Cells were incubated in this medium (300µl) for another 24 hours and equilibrated by incubation for another 24 hours. The media was then changed to the equilibration media either with or without 20µg/ml ApoA-I (Calbiochem), 4mg/ml 22(R)-hydroxycholesterol (Steraloids), and 10mM 9(cis)retinoic acid (Sigma). Efflux was quantified using a Microbeta plate reader (Wallac), and the data was calculated by dividing the counts in the media by the total counts of the media and the cells. Efflux values are an average of three wells per experiment with each experiment repeated four times.

Lipid analysis
Serum and lipoprotein lipid (cholesterol, triglycerides) concentrations were determined by enzymatic assays using commercially available reagents (Boehringer Mannheim, Germany). Serum HDL-cholesterol levels were determined after precipitation of ApoB-containing lipoproteins with phosphotungstic acid/Mg (Roche Diagnostics GmbH, Mannheim, Germany). Serum levels of ApoA-I, ApoA-II, ApoB and ApoC-III were measured by an immunonephelometric assay using
Table 1. Expression of ABCA1 in BAC+ApoE-/- mice

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Figure 1. Expression of ABCA1 in BAC+ApoE-/- mice

Increased ABCA1 protein was detected in the liver, kidney, brain, aorta, gonad, large intestine (Lg. Int.), and in peritoneal macrophage cells isolated from the BAC+ApoE-/- mice (A). An anti-ABCA1 monoclonal antibody raised against the second nucleotide binding domain was used (top panel) to detect ABCA1. To ensure equal protein loading, the Western blots were probed with anti-GAPDH antibody (lower panel). Human specific ABCA1 transcript was detected in all tissues tested (B) using RT-PCR. For each tissue, lane 1 is human specific, lane 2 is mouse specific, and lane 3 is an 18S control. The last panel is from a control mouse and contains no human transcript.

Specific mouse polyclonal antibodies as previously described (21).

HDL lipoprotein (d = 1.063-1.21 g/ml) and lipid-free protein (d > 1.21 g/ml) fractions were isolated from serum by sequential ultracentrifugation (22). The HDL fraction was assayed for its protein (23) and lipid (cholesterol, triglyceride and phospholipid) content. The apolipoprotein composition of ultracentrifugally isolated fractions was analyzed by polyacrylamide gradient gel electrophoresis under denaturing (0.1% SDS) and nonreducing conditions on ready-to-use gels (4-20 %, Novex, San Diego, CA) as described (24). Gels were stained with Coomassie Brilliant
Blue R-250 or transferred to nitrocellulose membranes. Replicates were used for immunochemical detection of murine ApoA-I and ApoA-IV using specific antibodies and images scanned using a densitometer (GS-300 scanning densitometer, Hoefer Scientific Instruments, CA).

Cholesterol efflux in response to serum lipid fractions
The capacity of serum lipid fractions to stimulate cellular cholesterol efflux was determined using Fu5AH rat hepatoma cells, following the procedure described by de la Llera Moya et al. (25). 50,000 Fu5AH cells were plated after which cellular cholesterol was labeled with 'H-cholesterol (NEN, Dupont de Nemurs, Paris, France) (1μCi/well). The cells were subsequently washed with PBS and incubated with either a 5% dilution of whole mouse serum or 50μg/ml HDL (density between 1.063 and 1.21g/mL) isolated from BAC+ApoE-/- and ApoE-/- control littermates. The medium was removed and centrifuged, and the cell monolayer washed and harvested. Radioactivity was then measured in both medium and cells, and the percentage of cholesterol efflux calculated. Efflux values are averages of four determinations, each performed in triplicate.

Statistical analysis
Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc.) version 2.0. The two tailed unpaired Student's t-test was used for all analyses, with a p value for significance set at 0.05.

Results
Increased ABCA1 protein levels in tissues of BAC+ApoE-/- mice
Increased ABCA1 protein levels were observed in the liver, kidney, brain, aorta, gonads and large intestine of the ABCA1 BAC+ApoE-/- mice, similar to data previously shown (17) for the ABCA1 BAC+ transgenic mice (Figure 1A). ABCA1 protein levels were also increased in peritoneal macrophages from the BAC+ApoE-/- mice compared to ApoE-/- littermates as previously shown for the BAC+ mice (17). All protein levels were assessed by densitometry with GAPDH used as a control.

Human ABCA1 transcript is expressed in tissues
Using human specific RT-PCR, human transcripts were detected in the liver, kidney, brain, aorta, gonads, large intestine, brain, heart and macrophages of the transgenic mice (Figure 1B).

ABCA1 BAC+ApoE-/- mice show increased cholesterol efflux from peritoneal macrophages
To examine whether the increased ABCA1 in the BAC+ApoE-/- mice was functional in the absence of ApoE gene expression, cholesterol efflux assays were performed on peritoneal macrophages. As shown for macrophages and fibroblasts isolated from the ABCA1 BAC+
transgenic mice (17), macrophages isolated from BAC+ApoE/- mice also showed increased efflux of cholesterol to ApoA-I compared to cells isolated from ApoE/- control littermates (n=4, p<0.001) (Figure 2). This efflux was further increased upon stimulation of the cells with 22(R)-hydroxy cholesterol and 9(cis)-retinoic acid indicating that even in the absence of the ApoE gene, the ABCA1 transgene is induced by specific activators of the LXR/RXR pathway, as previously shown (17). These data clearly show that normal ABCA1 activity is independent of ApoE expression within cells.

**ABCA1 BAC+ ApoE/- mice have mildly increased HDL-C levels**

A small increase in HDL-C levels was observed in the BAC+ApoE/- mice when compared to the littermates without the BAC transgene (0.25±0.07, n = 13, vs. 0.20±0.03, n = 11, p = 0.04) (Table 1). BAC+ ApoE/- mice displayed slightly elevated concentrations of serum total cholesterol when compared to ApoE/- littermates (p=0.04), as reflected by the increase in both HDL-C

**Figure 2.** Analysis of efflux levels in primary macrophages from BAC+ApoE/- mice

Primary peritoneal macrophage cultures were established from the BAC+ApoE/- and control mice as described (n=4) (17). A 38.4% increase (p<0.001) in efflux in the BAC+ApoE/- macrophages is evident when compared to the ApoE/- littermates. Upon stimulation of the cultures with 9(cis)-retinoic acid and 22(R)-hydroxy cholesterol, there was a 23.7% increase (p<0.001) in cholesterol efflux between BAC+ApoE/- versus control littermates. There was a further 46.8% increase (p<0.001) in efflux in the BAC+ApoE/- macrophages after stimulation with 9(cis)-retinoic acid and 22(R)-hydroxy cholesterol when compared to unstimulated cells.

**Table 1.** Lipid profiles of ABCA1 BAC+ApoE/- and ApoE/- control mice

<table>
<thead>
<tr>
<th>Lipid Profile</th>
<th>BAC+ ApoE/- (n = 13)</th>
<th>ApoE/- (n = 11)</th>
<th>p-value</th>
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<tr>
<td>TC (g/L)</td>
<td>7.49 ± 1.25</td>
<td>6.52 ± 0.83</td>
<td>0.04</td>
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<td>Non HDL-C (g/L)</td>
<td>7.24 ± 1.23</td>
<td>6.32 ± 0.83</td>
<td>0.05</td>
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<td>HDL-C (g/L)</td>
<td>0.25 ± 0.07</td>
<td>0.20 ± 0.03</td>
<td>0.04</td>
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<tr>
<td>TG (g/L)</td>
<td>1.37 ± 0.39</td>
<td>1.18 ± 0.36</td>
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<tr>
<td>ApoAI (g/L)</td>
<td>0.17 ± 0.03</td>
<td>0.16 ± 0.03</td>
<td>0.42</td>
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<tr>
<td>ApoAII (g/L)</td>
<td>0.14 ± 0.04</td>
<td>0.12 ± 0.02</td>
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<tr>
<td>ApoB (g/L)</td>
<td>0.65 ± 0.13</td>
<td>0.55 ± 0.06</td>
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<td>ApoCIII (g/L)</td>
<td>0.67 ± 0.16</td>
<td>0.56 ± 0.08</td>
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Increased activity of abca1 protects against atherosclerosis

levels stated, and non-HDL cholesterol (p=0.05) (Table 1). These data were confirmed by FPLC analysis (data not shown).

Triglycerides were not influenced by the transgene, whereas ApoB (p=0.03) and ApoC-III (p=0.05) concentrations were slightly increased in the BAC+ ApoE/- mice. No significant differences in the plasma levels of ApoA-I and ApoA-II (Table 1) between the BAC+ ApoE/- mice and the control littermates were seen (confirmed by SDS-PAGE analysis, data not shown).

**BAC+ ApoE/- mice show a significant decrease in atherosclerotic lesions**

ApoE/- mice develop detectable lesions in the aortic sinus valve as early as 10 weeks of age (5). These lesions enlarge in size and progress to the aortic arch as the mice age. Aortas from 12 week old mice were harvested, the aortic valve region sectioned and the ORO positive stained areas measured (Figure 3A). A marked (2.7 fold) decrease in the lesion area was observed in the BAC+ApoE/- mice when compared to ApoE/- littermates (27.9mm² vs. 74.1mm², p<0.0001) (Figures 3A and B).

Moderately advanced atherosclerotic intimal lesions in the non-coronary and coronary sinuses of the ApoE/- mice (Figure 3D) were evident, limited primarily to the root of attachment of the valve cusps. The lesions in the aortic sinuses in the ApoE/- mice were more extensive than in the sinuses of BAC+ApoE/- mice, often being confluent from commissure to commissure. Advanced non-coronary aortic sinus lesions were enriched with foam cells, had an apparently intact overlying endothelium, and contained several mesenchymal cells within the deep intimal matrix. There were no observable atherosclerotic lesions in the coronary arteries. Prominent intimal ORO positivity was seen in all aortic sinuses of ApoE/- mice. However, much more lipid deposition, in the form of both micro and macro droplets, was observed in the non-coronary sinuses than in the coronary sinuses. In general, ORO staining was localized to foam cells. In the aortic sinuses from ApoE/- mice, there was extensive deep intimal matrix deposition (observed as a sea green color) toward the commissures. Also, extracellular cholesterol clefts were observed in these lesions, along with thinning of the walls of the sinuses of Valsalva. The beginning of a fibrous cap was observed in the more advanced lesions (Figure 3H).

In contrast to the ApoE/- mice, there was markedly reduced atherosclerosis in the aortic sinuses of BAC+ ApoE/- mice, a difference easily seen by the naked eye. Occasional small, early, focal peri-commissural foam cell-rich lesions in some non-coronary sinuses, and many small aggregates of foam cells in the intima of both non-coronary and coronary sinuses were evident. As in the ApoE/- mice, there was no involvement of the coronary arteries. ORO staining was markedly less in aortic sinuses of BAC+ ApoE/- mice (figure 3C). There was diffuse ORO positivity in foam cells in areas around the commissures. In Movat’s pentachrome stained sections from the BAC+ ApoE/- mice, there was only slight matrix enrichment in the noduli Aranti (Figure 3G). Further, only limited thinning of the sinus walls was observed, with no fibrous cap formation.

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Figure 3. Assessment of lesions in BAC+ApoE-/− mice
Lesions in aortic roots of BAC+ApoE-/− mice (A, left panel) and ApoE-/− (A, right panel) mice (5X) were stained with Oil Red O (ORO) to detect the accumulation of lipids. BAC+ApoE-/− mice showed a 2.7x decrease in lesion size compared to the ApoE-/− mice (3B). ORO stained sections were used for the assessment of lesions (3C and D) (24X, inset 4X of transversely sectioned aortic root). Lesions were markedly smaller and less well developed in BAC+ApoE-/− mice compared to ApoE-/− mice. Markedly less ORO positivity was seen in foam cells from BAC+ApoE-/− mice as compared to ApoE-/− mice. A color overlay based on hue, saturation and intensity further shows a dramatic decrease in ORO positivity (green color) in lesions from BAC+ApoE-/− mice (3E), compared to ApoE-/− mice (3F) (24X). For the assessment of lesion complexity in BAC+ApoE-/− mice, lesions in aortic roots of BAC+ApoE-/− mice (3G) and ApoE-/− mice (3H) were stained with Movat’s pentachrome to visualize extracellular matrix deposition within the lesions (24X, inset 4X of transversely sectioned aortic root). Small amounts of matrix rich in proteoglycans, especially versican (reflected here as sea green color corresponding to glycosaminoglycan content), is observed in lesions from BAC+ApoE-/− mice particularly near the noduli Arantii. Further, these early lesions have only a very faint intimal positivity. However, in lesions of the ApoE-/− mice, glycosaminoglycan is observed in deep intimal areas of the lesions underlying the foam cell rich regions. Also, extracellular matrix deposition is observed interwoven with extracellular cholesterol clefts and near the noduli Arantii. A color overlay reflecting extracellular matrix based on hue, saturation and intensity shows differential localization of these components (green color) in BAC+ApoE-/− (3I) and ApoE-/− mice (3J) (24X). An aortic valve cusp is denoted in (G) (arrow).

Table 2. Efflux to HDL and serum isolated from ABCA1 BAC ApoE-/− and ApoE-/− mice

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<th>Fu5AH (n = 4)</th>
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<td>HDL ApoE-/−</td>
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<tr>
<td>9.97±0.73</td>
<td>BAC+ ApoE-/−</td>
<td>13.27 ± 0.73</td>
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<td>Serum ApoE-/−</td>
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<tr>
<td>16.75 ±0.08</td>
<td>BAC+ ApoE-/−</td>
<td>18.32 ± 0.32</td>
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HDL and serum from BAC+ApoE/- mice display increased cholesterol efflux-stimulating capacity

The BAC+ApoE/- mice show dramatic reductions in atherosclerosis with only mild changes in lipid levels. This suggested that the protection against atherosclerosis may be mediated not only by changes in total lipid levels, but also influenced by changes in cellular function due to alterations in HDL composition. In support of this, various single nucleotide polymorphisms in humans may be associated with significant changes in susceptibility to atherosclerosis without obvious changes in total lipid levels (26). To determine whether serum and HDL isolated from BAC+ transgenic mice show an altered capacity to act as an efflux acceptor, the cholesterol efflux-stimulating capacity of diluted serum isolated from these mice was measured in Fu5A H cells, a well established and characterized system for measuring cholesterol efflux (25). Serum from the BAC+ApoE/- mice was more effective in inducing cholesterol efflux as compared to serum from the ApoE/- mice (18.32 ± 0.32 vs. 16.75 ± 0.08, p<0.0001) (Table 2). To assess whether this greater efflux-stimulating capacity of serum was due to changes in the HDL fraction, the capacity of HDL (density (d) between 1.063 and 1.210 g/ml) isolated from BAC+ApoE/- mice and control littermates to induce efflux was compared. The HDL from BAC+ ApoE/- mice displayed a 33 % higher capacity to induce efflux of cellular cholesterol than the HDL from control animals (13.27 ± 0.73 vs. 9.97 ± 0.73, p=0.0007). It is notable that the relatively minimum effect observed with Fu5AH cells may be due to the fact that these cells rely mostly on SR-B1 for cholesterol efflux (27).

HDL and lipid-free fractions from ABCA1 BAC+ApoE/- mice show alterations in composition

The enhanced capacity of HDL from BAC+ApoE/- mice to induce efflux suggested that these transgenic mice may show changes in HDL composition. Interestingly, the chemical composition

![Figure 4](image-url): Correlation between the phospholipid content of HDL and its ability to elicit efflux. HDL (d between 1.063 and 1.21 g/ml) isolated from BAC+ApoE/- and control ApoE/- mice was used for the quantification of phospholipid content and its ability to elicit cholesterol efflux. Phospholipid was quantified using a previously characterized method (26). Efflux was measured using Fu5AH cells, using protocols previously described (25). Linear regression analysis was performed on the % phospholipid content of the HDL and the % efflux elicited. The phospholipid content of HDL accounts for 68% of its ability to induce cholesterol efflux.
of HDL isolated by ultracentrifugation from plasma from the BAC-ApoE-/− mice show an increased phospholipid content (p=0.05) (Table 3). Linear regression analysis revealed a highly significant correlation between phospholipid content of HDL and its ability to induce efflux (r = 0.68, p = 0.02) (Figure 4). A concurrent slight decrease in protein content was observed in the HDL from BAC+ApoE-/− mice (59% vs. 64%, p = 0.04). No significant difference in the cholesterol content of the HDL was observed (26% vs. 25%, p = 0.42) (Table 3). Triglyceride concentrations of the HDL were not detectable.

In addition, ApoA-I and ApoA-IV levels were measured in the lipid free fractions with d>1.21g/ml. Since it has been shown that ultracentrifugation causes the loss of apolipoproteins from HDL fractions, we quantitated apolipoproteins from the lipid free fraction. A 15% increase in ApoA-IV levels (p = 0.017) in the BAC+ApoE-/− mice compared to control littermates was evident (Table 4). There was no significant difference in the ApoA-I levels.

### Table 3. HDL composition in ABCA1 BAC+ApoE-/− and ApoE-/− mice

<table>
<thead>
<tr>
<th></th>
<th>% Protein</th>
<th>% Phospholipid</th>
<th>% Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE-/−</td>
<td>63.6±2.74</td>
<td>11.4±1.99</td>
<td>24.9±0.97</td>
</tr>
<tr>
<td>BAC+ApoE-/−</td>
<td>59.0±2.22</td>
<td>15.3±2.49</td>
<td>25.6±1.34</td>
</tr>
<tr>
<td>p-value</td>
<td>0.04</td>
<td>0.05</td>
<td>0.42</td>
</tr>
</tbody>
</table>

### Table 4. ApoA-I and ApoA-IV levels in the lipid free fraction isolated from BAC+ApoE-/− and ApoE-/− mice

<table>
<thead>
<tr>
<th></th>
<th>ApoE-/−</th>
<th>BAC+ApoE-/−</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I</td>
<td>100±33</td>
<td>104±40</td>
<td>NS</td>
</tr>
<tr>
<td>ApoA-IV</td>
<td>100±24</td>
<td>115±23</td>
<td>0.017</td>
</tr>
</tbody>
</table>

### Discussion

A major unanswered question has been whether raised ABCA1 levels can confer protection against atherosclerosis. In this study we have used the ApoE-/− mice as a model for atherosclerosis and demonstrated that increased ABCA1 activity is associated with markedly reduced atherosclerosis. Not only was a decrease in lesion size evident, but also the increase in ABCA1 activity was associated with the appearance of earlier, less complex lesions with no fibrous caps and less foam cell involvement.

An immediately relevant issue is how increased ABCA1 activity protects against atherosclerosis. Increases in ABCA1 levels in the BAC+ApoE-/− mice result in significantly increased efflux of cholesterol from peritoneal macrophages. However, on the ApoE-/− genetic background, this increase in efflux is accompanied only by a small increase in plasma HDL-C levels. Interestingly, HDL isolated from the ABCA1 BAC+ transgenic mice was significantly more capable of inducing
Increased activity of abca1 protects against atherosclerosis

efflux from cells suggesting that qualitative as well as quantitative changes in HDL might contribute to its anti-atherogenic activity.
Interestingly, HDL isolated from the BAC-ApoE--/- mice contained significantly elevated phospholipid levels. ABCA1 has been proposed to be a phospholipid translocase/flippase (9;11), and one suggested mechanism of induction of efflux by ABCA1 is that it directly (28) or indirectly (29) forms a complex with ApoA-I, thereby causing the translocation of phospholipids to ApoA-I and leading to the formation of phospholipid-rich nascent HDL particles (30;31), which can then promote cholesterol efflux from macrophages and other tissues (32;33). Phospholipids are required for the formation of complexes between ApoA-I and cholesterol (34). Therefore ABCA1-mediated translocation of phospholipids to ApoA-I generates nascent HDL particles that are primed for further cholesterol efflux. Evidence in particular for this process is that in our mice, phospholipid content of the HDL was associated with 68% of the variation in efflux. The mechanism by which ABCA1 stimulates efflux is therefore both direct by translocating phospholipids and cholesterol to nascent HDL particles, and also indirect through changing the phospholipid content of HDL itself, further stimulating cholesterol efflux. The phospholipid content of HDL has a strong linear association with cholesterol efflux (35). Small changes in HDL composition are associated with large changes in total cholesterol levels (36). For example, HDL-phospholipid is decreased in patients with CAD and this is significantly correlated with increased severity of CAD (36).

Although there was no difference in ApoA-I and ApoA-II content in the HDL from both groups of mice, a significant 15% increase in ApoA-IV levels in BAC-ApoE--/- lipid free apolipoprotein fraction was evident. One of the functions of ApoA-IV determined in vitro is to promote cholesterol efflux from extrahepatic cells, partly because it is a potent activator of LCAT (37). it also serves as a ligand for HDL binding to hepatocytes (38). Raised ApoA-IV levels also offer protection against atherosclerosis. One mechanism that has been proposed is that ApoA-IV is a surrogate for ApoA-I containing particles in promoting reverse cholesterol transport (37) and may protect against atherosclerosis by mechanisms that are not directly related to HDL levels (39). ApoA-IV is a potent inhibitor of lipid oxidation, and oxidized LDL has an important role in the development of atherosclerosis. LDL oxidation is a prerequisite for its uptake by macrophages and the cellular accumulation of cholesterol (39). Thus, the elevated ApoA-IV levels that we observed in the BAC-ApoE--/- mice could itself confer additional protection against atherosclerosis. Taken together, our data suggest that ABCA1 plays a vital role in vivo, raising HDL levels and changing the composition of HDL, which facilitates the efflux of cholesterol from macrophages, leading in turn to a reduction in atherosclerotic lesions.

Joyce et al (40) have previously addressed the role of ABCA1 in atherosclerosis by generating ABCA1 cDNA transgenic mice crossed with ApoE--/- mice. Surprisingly, in contrast to our results, they showed a 2-2.6 fold increase in the size of lesions in the ABCA1xApoE--/- mice.
Chapter 4

The major difference between our two studies is that Joyce et al overexpressed the ABCA1 cDNA using the ApoE promoter containing its macrophage and hepatic control elements. In our study, on the other hand, ABCA1 was overexpressed using a construct containing endogenous regulatory elements (17, 41) to ensure that ABCA1 was expressed in a physiological manner in the proper cellular milieu and in response to the appropriate cell signals. ABCA1 has sterol responsive elements in its promoter region (42). These facts would argue that correct regulation in the appropriate cells is vital for the protective activity of ABCA1.

Using the ApoE promoter may lead to non-physiological cellular and subcellular expression of ABCA1. Indeed, in the manuscript by Joyce et al (40) there were only minimal non-significant changes in lipoproteins in the hABCA1×ApoE/- animals with no reported changes in efflux, suggesting that exquisite physiological regulation of ABCA1 by its natural promoter is essential for its normal function. These studies also suggest that ApoE and ABCA1 normally function in different subcellular compartments and confirm previously reported findings that ABCA1 is not normally dependant on ApoE for promoting efflux (43). In our animals, ABCA1 clearly promotes efflux in the absence of ApoE. Our study also shows that even though ApoE can function similar to ApoA-I as an acceptor for ABCA1 mediated efflux (44), the absence of ApoE as an acceptor is not rate limiting for the promotion of efflux and protection against atherosclerosis. The comparison between our two studies highlights the importance of using endogenous physiological promoters when addressing the role of a protein in a particular process, in this case, atherosclerosis. Also a recent report by Aiello et al (45) indicates that when ABCA1 is inactivated in macrophages in the ApoE/- background, there is markedly increased atherosclerosis and foam cell accumulation, providing further confirmation for the modulation of atherosclerosis as predicted by changes in ABCA1 expression (15-17).

In conclusion, our study demonstrates that raised ABCA1 activity results in a significant reduction in atherosclerotic lesion size and complexity, likely due to increased efflux and consequent changes in HDL-C levels and HDL composition.

Acknowledgements

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